[Document] Specification

[Title of Invention] Ligand Factor ERRL1 for Nuclear Receptor ERR, and Drug Screening Method

[CLAIMS]

[Claim 1] A mouse gene encoding a mouse protein ERRL1, which has the amino acid sequence of SEQ ID NO: 2 and functions as a ligand factor for a nuclear receotor ERR.

[Claim 2] A polynucleotide purified from genome DNA, mRNA or cDNA of the mouse gene of claim 1 or the complementary strand thereof, or a partial consecutive sequence thereof.

[Claim 3] The polynucleotide of claim 2, which consists of the nucleotide sequence of SEQ ID NO: 1 or a partial consecutive sequence thereof.

[Claim 4] A recombinant vector possessing the polynucleotide of claim 2 or 3.

[Claim 5] A transformed cell with the recombinant vector of claim 4.

[Claim 6] A mouse protein ERRL1, which has the amino acid sequence of SEQ ID NO: 2 and functions as a ligand factor for a nuclear receotor ERR.

[Claim 7] The protein ERRL1 of claim 6, which is an expression product of the polynucleotide of claim 2 or 3.

[Claim 8] A polypeptide or an oligopeptidem, which is a partial consecutive sequence of the amino acid sequence of SEQ ID NO: 2.

[Claim 9] A human protein having the same function as the mouse protein ERRL1 of claim 6.

[Claim 10] A human gene encoding the human protein of claim 9.

[Claim 11] A polynucleotide purified from genome DNA, mRNA or cDNA of the human gene of claim 1o or the complementary strand thereof, or a partial consecutive sequence thereof.

[Claim 12] An antibody agains the protein of claim 6 or 9.

[Claim 13] A transgenic mouse, which has the polynucleotide of claim 2 in its genome DNA and over expresses the protein ERRL1 of claim 6.

[Claim 14] A method of screening a substance which serves as the active ingredient in a drug for obesity and/or diabetes, which comprises treating cells or an animal with a candidate substance and specifying the candidate substance fulfilling one or more of the following requirements as the target active ingredient substance:

(a) increasing the expression level of a ligand factor ERRL1 for a nuclear receptor ERR;

(b) increasing the expression level of the nuclear receptor ERR;

(c) promoting the binding of ERRL1 to ERR; and

(d) increasing the expression level of an MCAD gene product.

[Detailed Description of the Invention]

[Technical Field of the Invention]

The invention of this application relates to a ligand factor ERRL1 for a nuclear receptor ERR (estrogen receptor- related receptor), and a novel method of screening a drug for obesity and/or diabetes by using the expression and the activity of the ERRL1 as an index.

[Background Art]

The most typical molecular mechanism that underlies gene expression regulated by nuclear receptors starts from the binding of their ligands (e.g., small lipophilic molecules such as steroids, retinoic acid, thyroid hormone and vitamin D_3) (Mangelsdorf, D. J. et al., Cell 83: 835-839, 1995). Endogenous levels of such ligands are strictly regulated by means of multiple enzymatic reaction steps working toward their production and/or degradation (Honkakoski, P. & Negishi, M. Biochem. J. 347: 321-337, 2000). In addition, these ligands are collectively called lipophilic ligands or lipophilic hormones of the

endocrine system. By changing ligand levels, the endocrine system contributes to the adaptation to changes in the in vitro or in vivo environment, namely to homeostasis (Mangelsdorf, D. J. et al., Cell 83: 835-839, 1995; Giguere, V. Endocr. Rev. 20: 689-725, 1999). This system appears advantageous for slow and long-term adaptations but disadvantageous for quick responses because of the complicated regulation of ligand production. On the other hand, genome analysis has predicted the existence of numerous nuclear receptor-like molecules. However, the lipophilic ligands specific to the molecules are currently hardly known, and these molecules are collectively called orphan receptors (Giguere, V. Endocr. Rev. 20: 689-725, 1999). The activation mechanisms of orphan receptors remain totally unknown. Estrogen receptor-related proteins 1 and 2 (ERR1 and -2) were the first identified orphan receptors (Giguere, V. et al., Nature 331: 91-94, 1988; Shigeta, H. et al., J. Mol. Endocrinol. 19: 299-309, 1997), and its third member (ERR3) has recently been isolated (Eudy, J. D. et al., Genomics 50: 382-384, 1998; Hong, H. et al., J. Biol. Chem. 274: 22618-22626, 1999). ERRs and estrogen receptors share structural similarity, however, ERRs do not respond to estrogen (Giguere, V. Endocr. Rev. 20: 689-725, 1999). On the other hand, ERR1 has been proposed to act as a key transcriptional regulator of the gene encoding medium-chain acyl CoA dehydrogenase (MCAD), namely a pivotal enzyme in mitochondrial fatty acid β-oxidation (Sladek, R. et al., Mol. Cell. Biol. 17: 5400-5409, 1997; Vega, R. B. & Kelly, D. P., J. Biol. Chem. 272: 31693-31699, 1997). These observations lead to the idea that ERR-mediated gene regulation may play important roles in the control of energy balance in the body by regulating fatty acid β-oxidation, which is generally induced by the physical exercises (Horowitz, J. F. & Klein, S., Am. J. Clin. Nutri. 72: 558S-563S, 2000). Therefore, daily performance of appropriate physical exercises is considered to be the simplest and most effective way to cope

with obesity and diabetes (Baldwin, K. M., J. Appl. Physiol. 88: 332-336, 2000). However, this concept has not yet confirmed due to lack of methodologies and findings for control of ERR-mediated gene expression.

Recently, by researchers in the nuclear receptor field, it has been revealed that several classes of transcriptional cofactor proteins, such as SRC1/p160 family (Onate, S. A. et al., Science 270: 1354-1357, 1995), P/CAF (Blanco, J. C. G. et al., Genes Dev. 12: 1638-1651, 1998), and CBP/p300 (Chakravarti, D. et al., Nature, 383: 99-103, 1996; Kamei, Y. et al., Cell 85: 403-414, 1996) play key roles in ligand-dependent transcriptional activation of the nuclear receptors. These are called coactivators and are ubiquitously expressed. Further, their expression levels appear not to change during differentiation of cells or in response to changes in external and internal environments. More recently, a unique coactivator, termed PPARγ-coactivator-1 (PGC-1) was identified (Puigserver, P. et al., Cell, 92: 829-839, 1998). This coactivator distinguishes itself from other coactivators by means of its tissue-specific and regulated expression. In other words, PGC-1 is expressed in different levels in brown adipose tissue (BAT), skeletal muscle, heart, kidney, and brain and the expression is markedly up-regulated in BAT after acute exposure to cold stress (Puigserver, P. et al., Cell, 92: 829-839, 1998). PGC-1 expression is also up-regulated in the liver (Yoon, J. C. et al., Nature, 413: 131-138, 2001) and heart (Lehman, J. J. et al., J. Clin. Invest. 106: 847-856, 2000) under fasting conditions.

PPARγ is known to be the key regulator of adipogenesis (Tontonoz, P. et al., Cell 79, 1147-1156, 1994), and its expression is augmented during the differentiation of adipocytes. However, the level of PGC-1 mRNA is at a very low level during adipocyte differentiation of 3T3-L1 cells. Therefore, it was speculated that a similar molecule to PGC-1,

which may function during adipocyte differentiation might exist.

[Problems to be solved]

As described above, a nuclear orphan receptor, ERR serves as a regulator for MCAD gene expression, and it is considered that the resistance to obesity or diabetes can be maintained by controlling energy balance in the body by regulating fatty acid β-oxidation by this MCAD gene expression. However, the ligand molecule for the receptor ERR which is the origin of a series of such mechanisms has not been identified yet.

Breakthrough of resistance mechanism for obesity or diabetes should be important to understand those clinical conditions and to develop treatment strategies. In addition, such breakthrough may strongly contribute to develop a novel drug for obesity or diabetes.

The invention of this application objects to provide a novel ligand factor for nuclear receptor ERR, and genetic manipulation materials thereof.

The invention of this application also objects to provide a a novel method of screening a drug for obesity and/or diabetes by using the expression and the activity of the ERRL1 as an index.

[Solutions for the problems]

This application provides the following (1) to (14) inventions.

(1) A mouse gene encoding a mouse protein ERRL1, which has the amino acid sequence of SEQ ID NO: 2 and functions as a ligand factor for a nuclear receotor ERR.

(2) A polynucleotide purified from genome DNA, mRNA or cDNA of the mouse gene of invention (1) or the complementary strand thereof, or a partial consecutive sequence thereof.

(3) The polynucleotide of invention (2), which consists of the nucleotide sequence of SEQ ID NO: 1 or a partial consecutive sequence

thereof.

(4) A recombinant vector possessing the polynucleotide of invention (2) or (3).

(5) A transformed cell with the recombinant vector of invention (4).

(6) A mouse protein ERRL1, which has the amino acid sequence of SEQ ID NO: 2 and functions as a ligand factor for a nuclear receotor ERR.

(7) The protein ERRL1 of invention (6), which is an expression product of the polynucleotide of invention (2) or (3).

(8) A polypeptide or an oligopeptidem, which is a partial consecutive sequence of the amino acid sequence of SEQ ID NO: 2.

(9) A human protein having the same function as the mouse protein ERRL1 of invention (6).

(10) A human gene encoding the human protein of invention (9).

(11) A polynucleotide purified from genome DNA, mRNA or cDNA of the human gene of invention (10) or the complementary strand thereof, or a partial consecutive sequence thereof.

(12) An antibody agains the protein of invention (6) or (9).

(13) A transgenic mouse, which has the polynucleotide of invention (2) in its genome DNA and over expresses the protein ERRL1 of invention (6)

(14) A method of screening a substance which serves as the active ingredient in a drug for obesity and/or diabetes, which comprises treating cells or an animal with a candidate substance and specifying the candidate substance fulfilling one or more of the following requirements as the target active ingredient substance:

(a) increasing the expression level of a ligand factor ERRL1 for a nuclear receptor ERR;

(b) increasing the expression level of the nuclear receptor ERR;

(c) promoting the binding of ERRL1 to ERR; and

(d) increasing the expression level of an MCAD gene product.

In this invention, 'polynucleotide' means a molecule in which phosphate esters of nucleosides in which a purine or a pyrimidine has been bound to a sugar via a β-N-glycosidic bond (ATP, GTP, CTP, UTP; or dATP, dGTP, dCTP or dTTP) have been bound to one another. Specific examples include a genomic DNA encoding a protein, an mRNA transcribed from the genomic DNA and a cDNA synthesized from the mRNA. It may be either a double strand or a single strand. Further, a sense strand and an antisense strand of these genomic DNA, mRNA and cDNA are included. In addition, 'protein' and 'peptide' mean a molecule composed of a plural number of amino acid residues bound one another via an amide bond (peptide bond).

The terms and concepts in this invention will be defined in detail in the description of the embodiments or Examples of the invention. In addition, various techniques used for implementing this invention can be easily and surely carried out by those skilled in the art based on known literatures or the like except for the techniques whose sources are particularly specified. For example, preparation of a drug is described in Remington's Pharmaceutical Sciences, 18th Edition, ed. A. Gennaro, Mack Publishing Co., Easton, PA, 1990, and techniques of genetic engineering and molecular biology are described in Sambrook, and Maniatis, in Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York, 1989; Ausubel, F. M. et al., Current Protocols in Molecular Biology, John Wiley & Sons, New York, N.Y, 1995 and the like.

[Embodiment for Carrying Out the Invention]

The invention (1) is a mouse gene encoding the ligand factor ERRL1, and this gene can be isolated by screening a mouse genomic DNA library using, as a probe, a DNA fragment consisting of a base sequence

of SEQ ID NO: 1 or a partial sequence thereof. The obtained genomic gene can be amplified by a gene amplification method, which is usually performed, such as the PCR (polymerase chain reaction) method, NASBN (nucleic acid sequence based amplification) method, TMA (transcription-mediated amplification) method or SDA (strand displacement amplification) method.

Thus amplified and isolated genomic DNA may be, for example, used for establishing a mouse (Knockout Mouse) of which ERRL1 is dysfunctioned. In addition, measurement of its transcriptional product or expression product may be an index for identifying a component of medicine for treating obesity or diabetes. Furhtermore, the mouse gene (genomic DNA) of the invention (1) includes an expression control regions (promoter/enhancer, suppressor) for the coding region of ERRL1 protein. Such expression control sequence may be useful as a material for searching a substance controlling expression of ERRL1 protein (transcriptional factor, etc.).

The invention (2) is polynucleotide (DNA fragment or RNA fragment) purified from the genomic gene of the invention (1), mRNA transcribed from the gene, cDNA synthesized from the mRNA. For example, cDNA can be obtained by preparing a cDAN library with a known method (Mol. Cell Biol. 2, 161-170, 1982 ; Gene, 150, 243-250, 1994) using poly(A)+RNA isolated from mouse cell as a template, and screening the cDNA library using a polynucleotide synthesized based on SEQ ID NO:1 as a probe. Alternatively, the cDNA can be obtained by RT-PCR method using pligonucleotides synthesized based on SEQ ID NO:1 as primers and mRNA isolated from mouse cell as a template. Suth obtained cDNA has a base sequence of SEQ ID NO:1.

The ERRL1 mRNA and cDNA include one encoding splicing variant of ERRL1. That is, the splicing variant lacks the region of 156Lue to 194Lys in SEQ ID NO:2, and a polynucleotide lacking 497-613

in SEQ ID NO:1 that correspond to the deletion region is included into the invention (2).

The polynucleotide of the invention (2) is a polynucleotide consisting of a partial consecutive sequence of the genomic gene of the invention (1), mRNA transcribed from the gene, cDNA synthesized from the mRNA. An embodiment of this polynucleotide is one consisting of a base sequence of coding region (ORF) in SEQ ID NO:1.

The polynucleotide may be used for genetic empirical production of ERRL1 protein as a form of a recombinant vector of invention (4).

The recombinant vector of invention (4) is a cloning vector or an expression vector, which can be optionally employed according to kinds of objects or polynucleotide. For example, in the case of producing ERRL1 protein using cDNA or its ORF as an insert, an expression vector for in vitro transcription, or an expression vector adequate for E. cili, prokaryotic cell such as Bacillus sabtilis, yeast, insect cell, eukaryotic cell such as a mammalian cell, respectively. BAC (Bacterial Artificial Chromosome) or cosmid vector may be also used for the genomic DNA of the invention (1).

For the transformant of invention (5), E. cili, prokaryotic cell such as Bacillus sabtilis, yeast, insect cell, eukaryotic cell such as a mammalian cell are employed in the case of producing ERRL1 protein using the recombinant vector of invention (4). These transformant may be prepared by introducing the recombinant vector into cell in accordance with a know method such as electroporation, calcium phosphate method, liposome method, DEAE dextran methd.

The ERRL1 protein of invention (6) is a purified mouse protein that consists of amino acid sequence of SEQ ID NO:2, and functions as a ligand molecule for nuclear receptor ERR. The term "function as a ligand molecule" means that the ERRL1 protein specifically binds to receptor ERR and facilitates a function of ERR (for example,

transcriptional control of MCAD gene expression). The ERRL1 protein includes its splicing variant, which is a protein deletes 156Leu to 194Lys in SEQ ID NO:2.

The ERRL1 protein can be obtained by a method of isolating from mouse cell or a method of preparing peptide using chemical synthesis based on the amino acid sequence of SEQ ID NO: 2, and preferably, can be produced by genetic engineering method using the polynucleotide of invention (2) or (3). That is, ERRL1 protein isolated from in vitro transcriptional system or transformed cell culture.

To produce ERRL1 ACE by in vitro translation, the polynucletide described above is inserted in the vector carrying RNA polymerase promoter to construct an recombinant vector. RNA for ERRL1 is produced by in vitro trancription using RNA polymerase, such as T7, T3, or SP6. These RNA molecules are incubated with rabbit reticrocyte lysate or wheat germ extract that contains materials for in vitro translation. As a promoter, pKA1、pCDM8、pT3/T7 18、pT7/3 19、pBluescript II are exemplified.

To produce ERRL1 in prokaryotic cells such as *E. coli*, the polynucletide described above is inserted in the vector carrying replication origin, promoter, ribosomal binding site, multiple cloning sites, and terminating site that could be worked in cognate prokaryotes. Recombinant protein products are extracted from cultured cells. For expressing vector for *E. coli*, pUC, pBluescripts II, pET or pGEX sytems could be used.

To produce ERRL1 in eukaryotes, the polynucletide described above is inserted in the vector carrying promoter, multiple cloning sites, splicing sequences, poly-A additional sequences, such as pKA1, pCDM8, pSVK3, pMSG, pSVL, pBK-CMV, EBV vector, pRS, pcDNA3, pYESs. Recombinant protein products were expressed in transformed cells. All eukaryotic cells could be avilable, while mammalian cells, such as COS7

and CHO, yeast cells, such as *Saccahromyces cereviciae,* and *Schizosaccahromyces pombe*, silkworm cells or *Xenopus laevis* oocytes are frequently used. For introducing expression vectors in eukaryotic cells, methods such as electroporation, calcium-phosphate, liposome or DEAE-dextran systems could be utilized.

For purifying recombinant ELL1 products, procedures combining established methods, such as treatments with detergents, sonication, treatments with enzymes, salt precipitation, dialysis, centrifugation, limiting filtration, gel filtration, SDS-PAGE, isoelectrofocusing gel electrophoresis, ion-exchanging chromatography, hydrophobic chromatography, affinity chromatography or reverse-phase chromatography, could be utilized.

The protein expressed in cell may be variously modified in cell. Such modified proteins would be included in a scope of the invention. The post-transcriptional modification includes deletion of N-end methionine, acetylation, glycosylation, limited degradation by intracellular protease, myristoylation, isoprenylation, phosphorylation, ubiquitination, ADP-ribosylation and methylation.

Furthermore, the protein of this invention includes a fusion protein with other protein or peptide. Those are, for example, a fusion protein with GST or GFP. Also, ERRL1 protein having Flag or His tag for easy purification would be included in the scope of this invention.

Thus obtained ERRL1 protein may be useful as a target molecule for searching a lead compound which will be a effective substance of a medicine for diabetes or obesity. It also may be an antigen for producing an antibody against ERRL1 protein.

The invention (8) is a polypeptide or oligopeptide consisting of a partial sequence of amino acid sequence of SEQ ID NO:2. The polypeptide or oligopeptide has the same amino acid sequence as the protein fragment corresponding to active region of ERRL1 protein, and

can be produced by a method of chemically synthesizing peptide, a genetic engineering method using a partial polynucleotide, or a method by cleavating ERRL1 protein of inventions (6) or (7) with an appropriate protease.

The polypeptide or oligopeptide may be also used for producing an antibody against the ERRL1 protein.

The invention (9) is a human protein (h-ERRL1 protein) having the same function as the muse ERRL1 protein. The protein functions as a ligand for human receptor ERR, and is homologous over 50%, preferably over 75%, more preferably over 90%, and most preferably over 95% to the amino acid sequence of SEQ ID NO:2. The h-ERRL1 can be obtained as a expression product of the human genomic gene of the invention (10). The genomic DNA of the invention (10) can be obtained by screening a human genomic library by using a purified polynucleotide or oligonulecotide consisting of base sequence of SEQ ID NO: 1 or a partial sequence thereof as a probe. The human-derived polynucleotide can be obtained by a method of screening a cDNA library using a probe synthesized based on the SEQ ID NO:1, or a method of RT-PCT using oligonucleotide synthesized based on SEQ ID NO:1 as primers and mRNA from human cell as a template.

The invention (12) is a a polyclonal antibody or a monoclonal antibody that recognizes ERRL1 protein, and includes a whole molecule that can bind to the epitope of the ERRL1 protein, and all the fragments of Fab, $F(ab')_2$, Fv and the like. Such an antibody can be obtained from serum after immunizing an animal by using a purified ERRL1 protein, or a partial peptide thereof as an antigen. Alternatively, it can be prepared by introducing an expression vector for a eukaryotic cell into the muscle or the skin of an animal with an injector or a gene gun, and collecting the serum. As the animal, mouse, rat, rabbit, goat, chicken or the like is used. If hybridoma is produced by fusing B cells collected from the

spleen of an immunized animal with myeloma, a monoclonal antibody can be produced.

Thus obtained antibody may be used for measuring an expression of ERRL1 protein, for example.

The transgenic mouse of invention (13) is a mouse obtained by developing a totipotent cell of being introduced with a polynucleotide encoding ERRL1 protein, and an offspring thereof. The transgenic mouse has the polynucleotide in chromosomes and highly expresses the exogenous ERRL1 protein in somatic cells. The transgenic mouse can be produced in accordance with a known transgenic animal production method (e.g., Proc. Natl. Acad. Sci. USA 77; 7380-7384, 1980). In other words, the foregoing polynucleotide (hereinafter, referred to as 'introduced gene' in some cases) is introduced into a totipotent cell of mouse and this cell is developed until it becomes an individual. Then, by selecting an individual in which the introduced gene has been integrated into the genome of the somatic cell, the target transgenic mouse can be produced.

In addition, a promoter sequence or an enhancer sequence is connected to the introduced gene to control its expression. Depending on the selection of this promoter/enhancer sequence, ERRL1 protein can be expressed systemically or it can be selectively expressed in a specific tissue.

Such an introduced gene can be constructed by introducing and ligating the foregoing polynucleotide and promoter/ enhancer sequence to a circular DNA vector so as to be arranged in an effective position in the expression regulation of the introduced gene. Then, after this vector DNA is digested with a restriction enzyme, the one, from which the vector region has been removed, is introduced into a totipotent cell.

As the totipotent cell to which a gene is introduced, a fertilized egg or an early embryo can be used. In addition, as the method of

introducing a gene into a totipotent cell, when a ratio of generating transgenic animal individuals or a ratio of inheriting an introduced gene to the next generations are taken into account, a physical injection (microinjection) method of DNA is the most preferred.

A fertilized egg, to which a gene is injected, is then transferred to the oviduct of a surrogate mother and after it is generated to an individual and born, the animal is grown with a foster parent and DNA is extracted from part of the body (e.g., tail tip), and the existence of the introduced gene is confirmed by the Southern analysis or the PCR method. Assuming that the individual in which the existence of the introduced gene has been confirmed is the first generation (founder), the introduced gene is inherited to 50% of its offspring (F1). Further, by crossing this F1 individual with a wild-type animal or other F1 animal, an individual (F2) having the introduced gene in one (heterozygote) or both (homozygote) of diploid chromosomes can be produced.

The transgenic mouse produced as above overexpresses ERRL1 protein in all somatic cells or a specific tissue and, as described in the after-mentioned Examples, it has a unique characteristic of being lean although it is hyperphagic, and showing significantly high energy expenditure. Such a transgenic animal is used in a screening method as describe below.

The invention (14) is a method of screening a substance which serves as the active ingredient in a drug for obesity and/or diabetes. The method comprises treating cells or an animal with a candidate substance and specifying a candidate substance fulfilling one or more of the following requirements as the target substance:

(a) increasing the expression level of a ligand factor ERRL1;

(b) increasing the expression level of a nuclear receptor ERR;

(c) promoting the binding of ERRL1 to ERR; and

(d) increasing the expression level of an MCAD gene product.

In the methods of the invention (14), a 'candidate substance' includes an organic or inorganic substance, a protein, a peptide, a polynucleotide, an oligonucleotide and the like, which are either unknown or known. 'Cells' are ones isolated from an established human cell line or non-human animal cell line or from a tissue of a human or a non-human animal body and appropriately maintained in a culture condition. Examples of the established cell line include an adipocyte such as 3T3-L1 cell line. As described in Examples below, because the expression of ERRL1 protein shows a sensitive response during the differentiation of this adipocyte, those are preferred cells for the search for a target substance. Alternatively, the cells may be an established cell line or bacteria (E. coli or the like) transfected with ERRL1 cDNA and/or ERR cDNA. In addition, as the cells isolated from an animal body, those of isolated from BAT, heart, skeletal muscle, kidney or the like, in which ERRL1 protein has been highly expressed, can be exemplified. On the other hand, "animal" is a preferably a mouse, and a candidate substance is administrated to this animal body (systemically or locally) or is taken as food or drink, then the foregoing requirements (a) to (d) are measured in a tissue or cells of the animal. At this time, a transgenic mouse of the invention (13) may be used as a positive control. In other words, because this transgenic mouse overexpresses ERRL1 protein and, as a result, highly expresses receptor ERR and MCAD genes, therefore, by measuring whether or not a wild-type mouse administered with a candidate substance fulfills the foregoing requirements as much as this transgenic mouse does, an effect of the candidate substance can be judged more precisely. In addition, in the case of using a animal as a target, a treatment of feeding with a high-fat diet, loading with physical exercises or the like, or amount of food intake, body weight, energy expenditure or the like can be included in the judging requirements.

The judgment of the foregoing requirements (a) to (d) can be

carried out by measuring the expression of the respective genes by measuring the level of their transcriptional products (mRNA or protein) using a known method. For example, a quantitative RT-PCT, Northern blotting and a Western blotting using an antibody against each expression product may be employed.

By using a active ingredient identified by the method, it will be possible to develop a drug for obesity and diabetes. That is, this drug has the actions selected from, firstly, the action of increasing the expression level of ERRL1, secondary, the action of increasing the transcriptional activity of ERR, and thirdly, the action of increasing the interaction between ERRL1 and ERR. Such type of drug is called as "exercise-mimic drug", and it is expected to have no or less side effects. This is because that exercise is the most riskless method for treating obesity and diabetes.

Hereunder, the invention of this application will be explained in more detail and specifically by showing Examples, however, the invention of this application is not intended to be limited to the following examples.

[Examples]

1. Methods

1.1. Database search

An EST homology search was performed using the BLAST program (Altshul, S. F. et al., Nucleic Acids Res. 25: 3389-3402, 1997).

1.2. RNA analysis

Northern blot analyses were performed in accordance with the description in the literature (Sambrook and Maniatis, in Molecular Cloning-A Laboratory Manual, 7.2-7.87, Cold Spring Harbor Laboratory Press, New York, 1989). The cDNA probes for MCAD (Genbank accession No. U07159), PRC (BC013720), ERR1 (U85259), ERR2 (S82458), ERR3 (AF117254), ACC2 (AF290178), UCP-1 (U63419) , UCP-3

(AF032902), CS (056479) and LDH (X51905) were obtained by RT-PCR.

1.3. Assays for transcriptional activity

Transfections and reporter assays were performed in accordance with the description in the literature (Takada, I. et al., Mol. Endocrinol, 14: 733-740, 2000). More specifically, a reporter gene containing four copies of a GAL4 binding sequence ($(UAS)_4$-Luc) was transfected into CV-1 cells in the presence or absence of a chimeric receptor expression vector in which the DNA binding region of GAL4 had been fused with the ligand binding region for the nuclear receptor (pCMX-GAL4-nuclear receptor), and the transcriptional activity of GAL4-nuclear receptor-fused protein was measured (Takada, I. et al., Mol. Endocrinol, 14: 733-740, 2000). All luciferase activities were normalized by the co-transfected β-galactosidase activities. Amino acids 1-147 of GAL4 fused to the ligand binding domain for the following nuclear receptors were used: mouse AR (amino acids, 607-899, Genbank accession number, X59592), human ERα (a.a. 251-595, X03635), human GR (a.a. 489-777, M10901), rat FXR (a.a. 190-469, U18374), human RARα (a.a. 126-432, X06538), human RXRα (a.a. 222-462, X52773), mouse PPARα (a.a. 156-468, X57638), human PPARγ1 (a.a. 176-478, L40904), human PXR (a.a. 110-434, AF084645), human ERR1 (a.a. 147-422, L38487), human ERR2 (a.a. 171-433, X51417), human ERR3 (a.a. 173-436, AF058291), human HNF4α (a.a. 125-465, X76930), human NOR (a.a. 361-626, D78579), human NURR1 (a.a. 264-535, S77154), human RORα1 (a.a. 140-523, U04897), human SF1 (a.a. 64-461, U76388), human COUP (a.a. 156-423, X12795), human TR2-11 (a.a. 149-603, M29960), human RevErbA (a.a. 199-614, M24898). The abbreviations of nuclear receptors are described in the references of respective Genbank files (Giguere, V., Endocr. Rev. 20: 689-725, 1999). GAL4-nuclear receptor expression plasmids were provided from Dr. K. Umesono. Mouse PGC-1 cDNA was obtained by screening a mouse embryo cDNA library. The full length cDNA of

human ERR1, 2 and 3 were obtained by RT/PCR. The amplified products were subcloned into pCAGGS and pCMX expression vectors, and confirmed by sequencing.

1.4. Protein interaction assays

In accordance with the description in the literature (Kamei, Y. et al., Cell 85: 403-414, 1996), the constructs (expression vectors) of GST fused with ERR1 and ERR3 were incubated with ^{35}S-ERRL1 (TNT, Promega) and washed. Then, the bound ^{35}S-ERRL1 was separated by SDS-PAGE, and quantified by autoradiography.

1.5. Stable cell lines

Phoenix 293 cells (gifted from Dr. G. P. Nolan, University of Stanford) were used for the retrovirus packaging (Grignani, F. et al., Cancer Res. 58: 14-19, 1998). pLNCX-derived expression plasmid (Clontech) containing cDNA of ERR1, ERR3 or GFP (control) and pMX-derived expression plasmid (Misawa, K. et al., Proc. Natl. Acad. Sci. USA 97: 3062-3066, 2000) containing cDNA of ERRL1, PGC-1 or GFP were used in accordance with the manufacturer's instruction.

1.6. Generation of transgenic mice

ERRL1 cDNA was cloned into pCAGGS (Niwa, H., Gene 108: 193-200, 1991), the transgene (Fig. 4A) was excised and purified (2 ng μl^{-1}). Fertilized eggs were recovered from BDF1 females (C57BL/6xDBA/2) crossed with BDF1 males and microinjected with the transgene by the standard method (Gordon, J., in Guide to Techniques in Mouse Development, (eds Wassarman, P. M. & DePamplilis, M. L., 747-771, Academic press, San Diego, 1993). Care of the mice was performed in accordance with the guideline of the institution to which the inventors belong.

1.7. High-fat diet

Mice were fed with a regular chow diet (Oriental Yeast Inc., Tokyo, Japan) or a high-fat diet (Aoki, N. et al., Obesity Res. 1: 126-131, 1993)

which contained casein (20% wt/wt), α-cornstarch (30.2%), sucrose (10%), lard (25%), corn oil (5%), minerals (3.5%), vitamins (1%), cellulose powder (5%), and D,L-methionine (0.3%).

1.8. Determination of energy expenditure

Oxygen consumption and carbon dioxide production were determined by using an indirect calorimeter system composed of a mass analyzer and a computer (Komenani, N. et al., J. Nutr. Sci. Vitaminol. (Tokyo) 41: 395-407, 1995). Mice were individually placed in an open-circuit plastic respiration chamber (24 x 46 x 18 cm) connected to a gas mass spectrometer (WSMR-1400, Westron, Chiba, Japan). The airflow was controlled at 2 l/min. Gas analysis was carried out from 10:00 to 9:00 on the following day. Samples were monitored continuously in 2 minute blocks in room air as a reference. Locomotor activity was recorded automatically every 10 minutes by an Animex-III (Shimadzu, Kyoto, Japan) laid under each respiration chamber. Energy expenditure was calculated in accordance with the description in the literature (Komenani, N. et al., J. Nutr. Sci. Vitaminol. (Tokyo) 41: 395-407, 1995).

1.9. KKAy mice

KKAy mice were purchased from Clea Japan Inc. (Tokyo).

1.10. In-door running trainer (treadmill)

Male mouse of 8 weeks old (C57BL/6) were run on treadmill (upslope of 10%, 15 m/min^{-1}). After running, the mouse were sacrificed at a given time, and RNA isolated from skeletal muscle (quadriceps muscle) was analyzed.

1.11. Statistical analyses

Statistical comparisons of data from two experimental groups were made by using Student's t-test. Comparison of data from multiple groups was made by one-way analysis of variance (ANOVA), and each group was compared with the other groups by the Fisher's protected

least significant difference (PLSD) test (Statview 4.0, Abacus Concepts). Statistical significance was defined as $P < 0.05$ or $P < 0.01$.

2. Results

2.1. Cloning of ERRL1 cDNA and its features

As a result of searching EST (expression sequence tags) for a PGC-1-related molecule, EST having an extremely high homology to PGC-1 was found, and the full length cDNA containing this EST was isolated. This cDNA consists of about 3.4 kb (SEQ ID NO: 1), and encodes a protein consisting of 1,014 amino acid sequences (sequence number 2). This protein was named ERRL1 (abbreviation of ERR ligand 1) based on its properties as a 'protein ligand' for ERRs (as shown later in detail). Incidentally, Lin et al. reported the cloning of a PGC-1 homologue named PGC-1β (Lin, J. et al., J. Biol. Chem. 277: 1645-1648, 2002), however, PGC-1β only has one amino acid difference from ERRL1 (The 260th leucine in the sequence number 2 is a proline in the PGC-1β). ERRL1 and PGC-1 show a high degree of amino acid identity except for the central unique region in ERRL1. The homologous region can be divided into five domains based on sequence identity and predicted functional properties (Fig. 1). The N-terminal region of ERRL1 (amino acids 1–282) contains two LXXLL motifs, which are a proposed binding motif to nuclear receptors (Torchia, J. et al., Nature, 387: 677-684, 1997; Heery, D. M., et al., Nature, 387: 733-736, 1997), and has 41% identity with PGC-1. The second region (amino acids 283-579), which is unique to ERRL1, contains E (glutamic acid) repeats and one LXXLL motif. The third region (amino acids 580–656) is highly conserved (47% amino acid identity). The fourth region (amino acids 657-882) is less conserved (22% amino acid identity) and contains a very short serine/arginine (SR)-rich domain (Tacke, R. & Manley, J. L. Curr. Opin. Cell Biol. 11: 358-362, 1999) in ERRL1 compared with the long SR domain in PGC-1. The C-terminal domain (amino acids 883-1014) has a

putative RNA-binding domain (Krecic, A. M. & Swanson, M. S., Curr. Opin. Cell Biol. 11: 363-371, 1999) and is highly conserved (52% amino acid identity).

2.2. Similar expression patterns between ERRL1 and ERR

The expression patterns of ERRL1 in different tissues from adult mice were examined. Two ERRL1 mRNAs of about 10 kb and 4 kb in length were observed. ERRL1 mRNAs were abundant in brain, BAT, heart and skeletal muscle, and were also detected in kidney, stomach and white adipose tissue (WAT). Consistent with a previous report (Puigserver, P. et al., Cell 92: 829-839, 1998), PGC-1 expression was increased in mouse BAT after exposure to cold stress, however, the expression of ERRL1 mRNAs was only marginally up-regulated by this stress.

Further, the expression pattern of ERRL1 closely resembles that of ERR1 (Sladek, R. et al., Mol. Cell. Biol. 17: 5400-5409, 1997 and Fig. 2), with both mRNAs being highly expressed in tissues that can use lipids as a source of cellular energy, e.g., BAT, heart, skeletal muscle, and kidney (Fig. 2A). ERR2 mRNA expression was not be detected, which is consistent with a previous study report (Giguere, V. et al., Nature 331: 91-94, 1988).

Then, it was examined whether ERRL1 expression is augmented during adipocyte differentiation of 3T3-L1 cells. It is certain that ERRL1 mRNAs were present at very low levels in 3T3-L1 preadipocytes and were markedly induced during adipocyte differentiation. In contrast, mRNAs of PGC-1 and PRC, another PGC-1-related molecule (Andersson, U. & Scarpulla, R. C. Mol. Cell. Biol. 21: 3738-3749, 2001) remained at low levels during this adipocyte differentiation (Fig. 2B). Similar augmentation of ERRL1 mRNAs was observed in mature adipocytes (Smas, C. M. & Sul, H. S. Biochem. J. 309: 697-710, 1995) differentiated from another preadipocytes, 10T1/2 cells (Fig. 2C). Further, expression

profiles of ERR1 and its target MCAD were quite similar to that of ERRL1 (Figs. 2B and 2C), however, ERR2 mRNA and ERR3 mRNA expressions were not detected in these cells. These results suggest that ERRL1 is involved in the gene regulations in mature adipocytes-related functions such as the lipid metabolism.

2.3. PGC-1 as a general nuclear receptor agonist.

It was examined that whether ERRL1 can function as a coactivator of PPARγ, using PGC-1 as a control coactivator. Contrary to our expectations, any evidence indicating that ERRL1 could activate PPARγ-mediated transcription could not be found out. In the control experiments, it was confirmed that the expression of PGC-1 on its own was able to drastically activate PPARγ-mediated transcription without the addition of any exogenous lipophilic ligands. PGC-1 is known to physically interact not only with PPARγ but also with several other nuclear receptors (Puigserver, P. et al., Cell 92: 829-839, 1998; Yoon, J. C. et al., Nature 413: 131-138, 2001; Tcherepanova, I. et al., J. Biol. Chem. 275: 16302-16308, 2000; Vega, R. B. et al., Mol. Cell. Biol. 20: 1868-1876, 2000; Delerive, P. et al., J. Biol. Chem. 277: 3913-3917, 2002). Furthermore, PGC-1 has been shown to be induced by certain environmental changes such as cold exposure (Puigserver, P. et al., Cell 92: 829-839, 1998) and fasting (Yoon, J. C. et al., Nature 413: 131-138, 2001; Lehman, J. J. et al., J. Clin. Invest. 106: 847-856, 2000). These observations raise the possibility that the expression of PGC-1 alone can activate multiple nuclear receptor pathways. This putative character is analogous to classical lipophilic ligands for the nuclear receptors. To address this possibility, nuclear receptors whose DNA-binding domains were replaced by the GAL4 DNA-binding domain were used. By this replacement, the comparison of transcriptional activity profiles of multiple nuclear receptors with the same reporter (gene) such as $(UAS)_4$-Luc can be easily performed. Indeed, PGC-1 was able to activate

transcription by means of multiple nuclear receptors including orphan HNF4α, SF1, and ERRs. Among these, PGC-1 most strongly activated HNF4α, which is consistent with a recent report (Yoon, J. C. et al., Nature 413: 131-138, 2001). Subsequently, PGC-1 activated ERα, SF1, ERRs, PPARs, PXR, RARα, and RXRα (Fig. 3A). From these observations, it was confirmed that PGC-1 can function as a general and broad nuclear receptor agonist.

2.4. ERRL1 as an ERR protein ligand

Using the same GAL4-fused nuclear receptor set as above, a potential partner of ERRL1 was searched, and it was found out that ERRL1 specifically activated ERR-mediated transcription. ERR3 was most strongly activated by ERRL1, followed by ERR1 and ERR2 (Fig. 3B). Then, the transcriptional activation properties of ERRL1 on full-length ERRs were tested. Consistent with the experiments above, ERRL1 dose-dependently activated the full-length ERR3- and the full-length ERR1-mediated transcription by means of the ERR responsive sequence (ERRE) of the MCAD gene promoter (Sladeck, R. et al., Mol. Cell. Biol. 17: 5400-5409, 1997; Vega, R. B. & Kelly, D. P. J. Biol. Chem. 272: 31693-31699, 1997) (Fig. 3C). The former was activated much more strongly than the latter.

Next, it was tested whether ERRL1 physically interacts with ERRs by in vitro binding analysis with glutathione S-transferase-fused ERR (GST-ERR) produced by using bacteria and ERRL1 translated in vitro. Matrix-bound GST-ERR1 and GST-ERR3, but not GST alone, retained radiolabeled ERRL1 efficiently (Fig. 3D). As reported previously (Sladek, R. et al., Mol. Cell. Biol. 17: 5400-5409, 1997; Vega, R. B. & Kelly, D. P. J. Biol. Chem. 272: 31693-31699, 1997), it was confirmed that ERRs had been bound to radiolabeled MCAD ERRE oligonucleotides in a gel mobility shift assay, and that the ERR-DNA complexes were supershifted by the addition of the ERRL1 protein. Given that lipophilic hormones

were not added either in the synthesis of the proteins or in the binding reactions, these results indicate that ERRL1 and ERRs can directly interact with the ERR target promoter DNAs.

Next, ERRL1 and ERR were expressed in 10T1/2 cell line, and it was examined whether ERRL1 can activate MCAD gene expression via the ERR. 10T1/2 cells were infected with several combinations of recombinant retroviruses, each containing a DNA encoding GFP (control), ERR1, ERR3, ERRL1 or PGC-1. Northern blot analysis was performed to confirm the expressions of the transduced genes in each cell line (Fig. 3E). The sole expression of either ERR1 or ERR3 led to a modest increase of MCAD mRNA level compared with that in cells infected with GFP alone. Coexpression of both ERR1 and ERRL1 or ERR3 and ERRL1 led to a further significant increase of the MCAD mRNA level (Fig. 3E). These results collectively demonstrate that ERRL1 can function as a 'protein ligand' for ERRs and activate ERR-mediated transcription at least in cultured cells.

2.5. Creation of ERRL1 mice

In order to examine the effects of increased ERRL1 expression in vivo, ERRL1 transgenic mice were created. It was expected that these mice would exhibit phenotypes that mimic those induced by activated ERR-mediated transcription. The chicken β-actin promoter with the cytomegalovirus immediate early enhancer (CAG promoter) was used to promote the expression of the mouse ERRL1 transgene in mice (Fig. 4A). Southern blot analysis of tail DNAs of the mice was performed to determine the transgene copy numbers (Fig. 4B).

Expression of the ERRL1 transgene was evaluated by Northern blot analysis on RNAs isolated from tissues of ERRL1 mice and control littermate mouse at 8 weeks of age (Fig. 4C). When a transgene-specific probe (Probe 2, Fig. 4A) was used, only a single 4-kb band was detected. The CAG promoter has been reported to show a strong activity in any

tissues (Niwa, H. et al., Gene 108: 193-200, 1991). Unexpectedly, however, the use of this promoter resulted in high expression levels of the ERRL1 transgene in several restricted tissues such as brain, BAT, heart, skeletal muscle, and testis, but a very low-level of expression in liver (Fig. 4C). These expression profiles corresponded relatively well to the endogenous ERRL1 expression patterns (Fig. 4C). These observations suggest that tissue-specific regulatory cis-sequences crucial for ERRL1 expression may reside in the ERRL1 cDNA used in this construct.

As predicted from the in vitro data, the increased expression of ERRL1 in the skeletal muscle of ERRL1 mice (Fig. 4D) enhanced MCAD mRNA expression in vivo. In contrast, the increased expression of ERRL1 did not affect the gene expression of acetyl-CoA carboxylase 2 (ACC2) (Abu-Elheiga, L. et al., Science 291: 2613-2616, 2001) or uncoupling protein-3 (UCP3) (Clapham, J. C. et al., Nature 406: 415-418, 2000).

2.6. ERRL1 mice are hyperphagic but lean

The apparent phenotype observed in ERRL1 mice was lean, which agreed well with those predicted from increased β-oxidation of fatty acids. This phenotype was more prominent when mice were fed with a high-fat diet (Aoki, N. et al., Obesity Res. 1: 126-131, 1993). Groups of six male wild-type control mice and six male ERRL1 mice, at 9 weeks of age, were freely fed with a high-fat diet, and the food consumption and body weight of each mouse were measured every week. ERRL1 mice consumed significantly more food than the control mice (Fig. 5A). However, the transgenic mice weighed 15 to 25% less than the control mice before and throughout the feeding periods with a high-fat diet (Fig. 5B), and they accumulated significantly less fat in their adipose tissues (Fig. 5C). The epididymal WAT in the ERRL1 mice only weighed 0.92 ± 0.28 g, compared with 2.0 ± 0.30 g in the control mice (Fig.). In contrast, liver

weights were not significantly different (ERRL1 mice: 1.23 ± 0.06 g; control mice: 1.4 ± 0.08 g). The adipocytes of the transgenic mice were smaller than those of the control mice (average diameter of adipocytes in ERRL1 mice: 25.1 ± 1.1 μm; control mice: 54.5 ± 2.2 μm; Figs. 5E and 5F).

The blood was collected from these mice, and serum components were biochemically analyzed. The decrease in adipose mass resulted in a decrease in the amount of leptin in the serum (ERRL1 mice: 5.9 ± 3.5 ng ml^{-1}, control mice: 25.0 ± 6.6 ng ml^{-1}). As leptin is an anti-appetite hormone secreted from WAT (Friedman, J. M. Nature 404: 632-634, 2000), the decreased level of leptin observed in ERRL1 mice is consistent with the observation that they are hyperphagic. Furthermore, decreased level of insulin were observed in the transgenic mice compared with the control mice fed with a high-fat diet. Mice, when fed with a high-fat diet, generally develop resistance to glucose uptake, even with a high plasma insulin concentration (Li, B. et al., Nat. Med. 6: 1115-1120, 2000). A similar state, which is called insulin resistance, is well observed in humans with obesity or who are suffering from type 2 diabetes (Lovejoy, J. C. Curr. Atheroscler. Rep. 1: 215-220, 1999). The decreased body weight and decreased insulin level observed in ERRL1 mice indicate that the increased expression of ERRL1 can antagonize obesity and contribute to an improvement of diabetic state, even taking a high-calorie diet through overcoming insulin resistance.

2.7. Increased energy expenditure of ERRL1 mice

The energy expenditure of ERRL1 mice was examined. Gas analyses were carried out by using test chambers for respiration analysis, each containing a single mouse, and energy expenditure was calculated. As a result, the energy expenditures of ERRL1 mice were significantly higher than those of control mice at 12 weeks of age (resting energy expenditure: ERRL1 mice, 126.3 ± 3.8 kcal day^{-1} $kg^{-0.75}$; control mice,

101.5 ± 6.7 kcal day^{-1} $kg^{-0.75}$; $P < 0.05$, n = 6, per group; and total energy expenditure: ERRL1 mice, 211.2 ± 10.7 kcal day^{-1} $kg^{-0.75}$; control mice, 155.9 ± 7.8 kcal day^{-1} $kg^{-0.75}$; $P < 0.01$, n = 6, per group) (Figs. 5G and 5H). However, locomotor activity was not significantly different (ERRL1 mice, 7,338 ± 700 counts; control mice, 5,338 ± 390 counts, the data represent the sum of all transits over a 24 h period). These results indicate that the differences in body weights between the control mice and the ERRL1 mice are most likely to be due to a difference in their energy expenditure.

2.8. ERRL1 expression can antagonize a genetically programmed obesity

ERRL1 mice and KKAy mice were bred, and it was examined whether the obese phenotype of KKAy mice was counteracted by the increased expression of ERRL1. KKAy mice have a mutation of Ay, which leads to ectopic expression of the agouti coat-color protein, causing a dominantly inherited syndrome of obesity and yellow fur (Siracusa, L. D., Trends Genet. 10: 423-428, 1994). The agouti protein acts as an antagonist for the melanocortine receptors, which are considered to act as a repressor receptor of appetite in the central nervous system (Friedman, J. M. Nature 404: 632-634, 2000; Siracusa, L. D., Trends Genet. 10: 423-428, 1994). KKAy males were bred with ERRL1 females, and their offsprings were selected by hair color (yellow, KKAy(+); black, KKAy(-)) and ERRL1 transgene expression. Comparison of the body weights of each group revealed that the ERRL1 transgene significantly suppressed the body weight increases of KKAy(+) mice (Figs. 6A and 6B), demonstrating that increased ERRL1 expression can antagonize obesity caused by a genetic abnormality.

2.9. Induced expression of ERRL1 after physical exercise

We searched conditions in which ERRL1 expression is induced in vivo. After numerous trials, we found that the ERRL1 expression was induced in the skeletal muscle when mice were forced to exercise on a

treadmill: the expression expressions of both ERRL1 and MCAD mRNAs were markedly increased by the exercise (Fig. 7A). In addition, mRNAs of citrate synthase (CS) and lactic acid dehydrogenase (LDH) were induced, which are also involved in the energy expenditure. An increase in the level of ERRL1 was well correlated to the enhancement of the MCAD, CS, and LDH expressions. Exactly same augmentation of MCAD, CS, and LDH expressions were observed in ERRL1 mice (Fig. 3D and 7B).

3. Discussion

From the above results, it was confirmed that ERRL1 functions as a 'protein ligand' for ERRs and controls energy expenditure in vivo. In other words, this indicates that by increasing the expression level of ERRL1 in, for example skeletal muscle, this functions as a ERR protein ligand this time, and activates MCAD gene expression to lead to activation of fatty acid β-oxidation. When fatty acid β-oxidation is increased, as a result, accumulated fat will be decreased. It is predicted that the same effects can be achieved by activating the transcriptional ability of ERR and by increasing MCAD gene expression. These effects are very similar to a physiological state which is achieved by physical exercises.

In addition, the above results clearly indicate that the body weight can be decreased by pharmacological activation of ERRL1 or ERR while maintaining the usual calorie intake or more.

Industrial Applicability

As described in detail above, according to the invention of this application, there are provided a novel ligand molecule ERRL1 to nuclear orphan receptor ERR, its genetic engineering materials, and a method of screening an ingredient in a remedy for obesity or diabetes by using the

interaction between a nuclear orphan receptor ERR and its ligand molecule ERRL1 as an index.

SEQUENCE LISTING

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905 910 915

gta gag tgc cag gtg ctg acg aga agt aaa aga ggc cag aag cac ggt 2836
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Ala Ser Asp Phe Asp Ser Ala Thr Cys Phe Gly Glu Leu Gln Trp Cys
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Pro Glu Thr Ser Glu Thr Glu Pro Ser Gln Tyr Ser Pro Asp Asp Ser
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Glu Leu Phe Gln Ile Asp Ser Glu Asn Glu Ala Leu Leu Ala Ala Leu
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Thr Lys Thr Leu Asp Asp Ile Pro Glu Asp Asp Val Gly Leu Ala Ala
100 105 110

Phe Pro Glu Leu Asp Glu Gly Asp Thr Pro Ser Cys Thr Pro Ala Ser
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Pro Ala Pro Leu Ser Ala Pro Pro Ser Pro Thr Leu Glu Arg Leu Leu
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Ser Pro Ala Ser Asp Val Asp Glu Leu Ser Leu Leu Gln Lys Leu Leu
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Leu Ala Thr Ser Ser Pro Thr Ala Ser Ser Asp Ala Leu Lys Asp Gly
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Ala Thr Trp Ser Gln Thr Ser Leu Ser Ser Arg Ser Gln Arg Pro Cys
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Val Lys Val Asp Gly Thr Gln Asp Lys Lys Thr Pro Thr Leu Arg Ala

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Gln Ser Arg Pro Cys Thr Glu Leu His Lys His Leu Thr Ser Val Leu

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245 250 255

Ser Pro Trp Leu Thr Pro Ala Ser Pro Gln Asp Ser Leu Ala Gln Asp

260 265 270

Thr Ala Ser Pro Asp Ser Ala Gln Pro Pro Glu Glu Asp Val Arg Ala

275 280 285

Met Val Gln Leu Ile Arg Tyr Met His Thr Tyr Cys Leu Pro Gln Arg

290 295 300

Lys Leu Pro Gln Arg Ala Pro Glu Pro Ile Pro Gln Ala Cys Ser Ser

305 310 315 320

Leu Ser Arg Gln Val Gln Pro Arg Ser Arg His Pro Pro Lys Ala Phe

325 330 335

Trp Thr Glu Phe Ser Ile Leu Arg Glu Leu Leu Ala Gln Asp Ile Leu

340 345 350

Cys Asp Val Ser Lys Pro Tyr Arg Leu Ala Ile Pro Val Tyr Ala Ser

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Leu Thr Pro Gln Ser Arg Pro Arg Pro Pro Lys Asp Ser Gln Ala Ser

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Pro Ala His Ser Ala Met Ala Glu Glu Val Arg Ile Thr Ala Ser Pro

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450 455 460

Lys Leu Gly Arg Lys Met Asp Ser Ser Val Cys Pro Val Arg Arg Ser

465 470 475 480

Arg Arg Leu Asn Pro Glu Leu Gly Pro Trp Leu Thr Phe Thr Asp Glu

485 490 495

Pro Leu Gly Ala Leu Pro Ser Met Cys Leu Asp Thr Glu Thr His Asn

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Leu Glu Glu Asp Leu Gly Ser Leu Thr Asp Ser Ser Gln Gly Arg Gln

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Leu Pro Gln Gly Ser Gln Ile Pro Ala Leu Glu Ser Pro Cys Glu Ser

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Gly Cys Gly Asp Thr Asp Glu Asp Pro Ser Cys Pro Gln Pro Thr Ser

545 550 555 560

Arg Asp Ser Ser Arg Cys Leu Met Leu Ala Leu Ser Gln Ser Asp Ser

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Glu Asp Pro Phe Lys Pro Asp Thr Lys Leu Ser Pro Gly Gln Asp Thr

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Leu Ser His Leu Gln His Ala Thr Thr Gln Pro Val Ser Gln Ala Gly

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Gln Lys Arg Pro Phe Ser Cys Ser Phe Gly Asp His Asp Tyr Cys Gln

675 680 685

Val Leu Arg Pro Glu Ala Ala Leu Gln Arg Lys Val Leu Arg Ser Trp

690 695 700

Glu Pro Ile Gly Val His Leu Glu Asp Leu Ala Gln Gln Gly Ala Pro

705 710 715 720

Leu Pro Thr Glu Thr Lys Ala Pro Arg Arg Glu Ala Asn Gln Asn Cys

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Asp Pro Thr His Lys Asp Ser Met Gln Leu Arg Asp His Glu Ile Arg

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Lys Arg Gly Gln Lys His Gly Phe Ile Thr Phe Arg Cys Ser Glu His

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Ala Ala Leu Ser Val Arg Asn Gly Ala Thr Leu Arg Lys Arg Asn Glu

945 950 955 960

Pro Ser Phe His Leu Ser Tyr Gly Gly Leu Arg His Phe Arg Trp Pro

965 970 975

Arg Tyr Thr Asp Tyr Asp Pro Thr Ser Glu Glu Ser Leu Pro Ser Ser

980 985 990

Gly Lys Ser Lys Tyr Glu Ala Met Asp Phe Asp Ser Leu Leu Lys Glu

995 1000 1005

Ala Gln Gln Ser Leu His

1010

[Brief Description of Drawings]

[Fig. 1]

The comparison of the primary amino acid sequences between ERRL1 and PGC-1. Asterisks indicate identical amino acids. The numbers represent the amino acid positions with the first methionine counted as one. LXXLL motifs are shown in bold types. Glutamic acid (E) repeats and serine-arginine (SR)-rich regions are underlined. A putative RNA-binding motif is boxed. Tentative domain borders are shown by vertical lines. A splicing variant of ERRL1, which was newly identified at this time, lacks 39 amino acids (from the 156th leucine to the 194th lysine).

[Fig. 2]

The expression profiles of ERRL1 mRNA. Fig. 2A shows the results of analyzing 20 μg of total RNA from various adult mouse tissues using ERRL1, PGC-1, PRC, ERR1 and ERR3 cDNAs as probes. Ethidium bromide staining of 28S RNAs is also shown. Fig. 2B shows the results

obtained by inducing 3T3-L1 cells to differentiate into adipocytes by the treatment with dexamethasone, 1-methyl-3-isobutylxanthin and insulin (added day 0), isolating RNAs and performing the Northern blot analysis. The blot was sequentially hybridized with the respective probes. Fig. 2C shows the results of observing the expression of ERRL1, ERR1 and MCAD mRNA in 3T3-L1 and 10T1/2 cells (-: preadipocytes, +: mature adipocytes).

[Fig. 3]

The profiles of PGC-1 and ERRL1 as 'protein ligands' for various nuclear receptors. Transcriptional activation ability of PGC-1 (A) and ERRL1 (B) for various nuclear receptors was examined in a transfection assay. Mean values of triplicate experiments are shown as fold induction, where the Luc activity of each GAL4-fused nuclear receptor in the absence of PGC-1 or ERRL1 serves as the reference values. Error bars show standard deviation. Fig. 3C shows the dose-dependent activity of ERRL1 on the full-length ERR-mediated transcription via ERRE. pCMX-ERRL1 in different amounts (0 to 200 ng) was co-transfected with 30 ng of pCMX-ERR1 or pCMX-ERR3 in the presence of 250 ng of TK-Luc or ERRE-Luc in CV-1 cells. Mean values from triplicate experiments are shown as fold induction, where the Luc activity of TK-Luc in the absence of ERRL1 serves as the reference value. Error bars show standard deviation. Fig. 3D shows the results of confirming that ^{35}S-labeled ERRL1 shows strong interaction with GST-ERR1 and GST-ERR3 in vitro. Autoradiographs show the pulled down proteins (GST-ERR1 and GST-ERR3) and 10% of the total amount of ^{35}S-labeled ERRL1 used for the GST pull-down assay (Input). CBB staining of the SDS-PAGE gel containing GST-fused proteins used in this assay is also presented. Fig. 3E shows the results showing augmentation of MCAD mRNA expression by ectopic expression of ERRL1 or PGC-1 in combination with ERR1 or ERR3 in 10T1/2 cells. One day

after confluence of the cells, total RNA was isolated, and analyzed by Northern blot (20 μg per lane) using MCAD and ERRL1 cDNAs as probes. The relative densitometric values of MCAD mRNAs are also included beneath each lane.

[Fig. 4]

The creation of ERRL1 transgenic mouse. Fig. 4A shows the schematic drawing of the ERRL1 transgene and the positions of probes used for Southern (probe 1) and Northern blots (probe 1 and probe 2). Fig. 4B shows the results of showing that in the two transgenic lines (A1 and A2), 10 and 12 copies of each of the transgenes were contained, respectively. Fig. 4C shows the results of Northern blot analysis of ERRL1 mRNA expression in the respective tissues from ERRL1 mice (line A1) and littermate control mice. Each lane contained 20 μg of total RNA. Fig. 4D shows the results of measuring the expression of ERRL1, MCAD, ACC2 and UCP-3 in the skeletal muscle of ERRL1 transgenic mice and wild-type control mice. Three mice in each group were examined. Quantitative values of Northern signals (control as 100%) are shown on the right. Data are mean ± s.e.m. (Asterisk shows $p < 0.05$).

[Fig. 5]

The phenotypes of ERRL1 transgenic mice. Fig. 5A shows the cumulative food intake per mouse during the indicated period by measuring food intake every week. Data are means ± s.e.m. ($n = 6$ per animal group, error bars are smaller than the symbols, asterisk shows $P < 0.05$, double asterisk shows $P < 0.01$). Fig. 5B shows the results of showing the changes in the body weight of ERRL1 transgenic mice (closed circle) and wild-type controls (open circle). Values represent mean body weight ± s.e.m. ($n = 6$ per animal group, asterisk shows $P < 0.05$, double asterisk shows $P < 0.01$). For some data points, the error bars are smaller than the symbols. Fig. 5C shows the results of reduction of abdominal fat in ERRL1 transgenic mice. Fig. 5D shows

the comparison of epididymal WAT weight between wild-type control mice and ERRL1 transgenic mice. The columns represent mean values of WAT weight ± s.e.m. (n = 6 per animal group, asterisk shows $P < 0.05$). Fig. 5E shows the comparison of the morphology of white adipose tissue between ERRL1 transgenic mice and littermate wild-type control mice. The scale bar indicates 50 μm. Fig. 5F shows the average diameter of adipose cells. The diameters of the cells were measured from the sections shown in Fig. 5E (n = 20, double asterisk shows $P < 0.01$). Figs 5G and 5H show the results of showing energy expenditures at resting (G) and total (H) in 12-week-old control mice and ERRL1 transgenic mice. The columns represent mean values of energy expenditure ± s.e.m. (n = 6 per animal group, asterisk shows $P < 0.05$, double asterisk shows $P < 0.01$).

[Fig. 6]

6A shows the results of measuring changes in body weight of KKAy(+)ERRL1(-) (open square, n = 8), KKAy(-) ERRL1(-) (open circle, n = 5), KKAy(+)ERRL1(+) (closed square, n = 5), KKAy(-)ERRL1(+) (closed circle, n = 5) male mice. The body weight curve of each group was compared by repeated-measure analysis (Super ANOVA). Asterisk shows $P < 0.05$, double asterisk shows $P < 0.01$. 6B shows representative photographs of 12-week-old male KKAy(+)ERRL1(-) (left), KKAy(+)ERRL1(+) (center) and KKAy(-)ERRL1(-) (right) mice.

[Fig. 7]

Augmentation of ERRL1, MCAD, CS, and LDH gene expression in response to exercise and in ERRL1 mice. A, Mice were either kept sedentary (control) or exercised by a treadmill run of 2 h. At indicated hours after the exercise, mice were sacrificed and skeletal muscle mRNA was isolated. Two mice in each time points were examined in the Northern blot analysis. B, ERRL1, MCAD, CS, and LDH expressions in the skeletal muscle of ERRL1 mice and wild-type controls. Three male

mice in each group were examined in the Northern blot analysis. Quantification of Northern signals (control as 100%) are shown on the right. See Figure 4D for MCAD expression. Data are mean ± s.e.m. (Asterisk; $p<0.05$, double asterisk; $p<0.01$).